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TITLE: QUALITY ASSURANCE OF SAMPLES AND PROCESSES IN THE SPANISH RENAL RESEARCH NETWORK (REDinREN) BIOBANK

RUNNING TITLE: QUALITY ASSURANCE IN THE REDinREN BIOBANK

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ABSTRACT

Biobanks are useful platforms to build bridges between basic, translational and clinical research and clinical care. They are repositories of high-quality human biological samples ideal for evaluating their histological characteristics and also their genome, transcriptome and proteome. The Spanish Renal Research Network Biobank contains more than 76,500 well-preserved frozen samples of a wide variety of kidney diseases, collected from 5,450 patients seen over 70 nephrology services throughout the Spanish territory. **Objective:** to determine and to report the results of the quality control of samples and processes conducted in our biobank, implemented in accordance with the requirements of ISO 9001:2008 international standard. **Study design:** two types of quality controls were performed: i) systematic i.e. measurement of viable Peripheral Blood Mononuclear Cells (PBMC) obtained and purity of nucleic acids and ii) ad-hoc: i.e. viability of thawed PBMC, DNA extraction process reproducibility and the integrity and functionality of nucleic acids, implemented on a routine basis. **Methods and Results:** PBMC isolation by Ficoll yielded reproducible results and their cryopreserved viability was greater than 90%. Acceptable A260/A280 ratios were obtained for the vast majority of the DNA (n=2328) and RNA (n=78) analyzed. DNA integrity was demonstrated by agarose gels and by β-globulin gene PCR amplification of 1327 and 989 bp fragments. . DNA of acceptable quality had at least three bands of β-globulin amplified obtained (n=26/30). RNA Integrity Number (RIN) determination obtained RIN numbers ≥ 7 (n=87/96). The ampliafiability of nucleic acids was confirmed by qPCR and RT-qPCR of β-actin and GAPDH genes. Long storage or delay processing time did not affect the quality of the samples analyzed. The processes of DNA extraction also yielded reproducible results. **Conclusions:** these results clearly indicate that our PBMC, DNA and RNA stored samples meet the required quality standards to be used for biomedical research, ensuring their long-term preservation.

KEY WORDS: biobank, ISO 9001:2008, quality control, chronic kidney disease

INTRODUCTION

Currently, the knowledge of the complex etiology of diseases constitutes a challenge, given the multitude of factors that may influence their development, which include genetic predisposition, lifestyle and environmental conditions. Knowing and understanding these complex interactions are critical to facilitate researchers' access to a large number of well-documented biological samples, selected according to scientific criteria well suited to the research to be developed and that meet the quality standards required for analysis using new high-performance technologies. In this context, it is highly useful to build easily accessible infrastructure that enables rapid experimental demonstration of a hypothesis or the verification of a simulation model. A biobank is a facility which processes, stores, archives and distributes human samples and their associated clinical data, which are used to promote the advance of biomedical research. Well-preserved frozen samples are ideal for evaluating the genome, the transcriptome, the proteome and the metabolome[1-3]. When the molecular analysis of nucleic acids, proteins, and metabolites is being conducted, the quality of the starting samples obtained from these repositories has to be taken into consideration, since factors such as degradation, the presence of impurities, and enzyme inhibitors may have a significant impact on the results obtained[4-9]. Hence, it is necessary to ensure the adequacy of samples available for mass screening analysis used at current translational research. These rigorous demands as well as the long-term storage of samples stored in biobanks require the implementation of quality controls; these controls will depend on the type of sample stored[5-14].

At present, there are many biobanks that conduct quality controls; and there is some consensus on the specific type of quality control to be performed on each different sample. In this regard, for RNA and DNA samples, it is important to check their integrity and purity[1-4; 6-12]. For RNA samples, the ratio of absorbance at 260nm and 280nm is used to assess their purity[15]. To determine RNA sample integrity, two major ribosomal bands, 28s and 18s, should be observed in agarose gel or microchip electrophoresis[16], the RNA Integrity Number (RIN) can be calculated[1], and genes can be amplified by RT-PCR[2]. The purity of DNA samples is assessed in the same way as the RNA samples[15], whereas its integrity can be obtained by observing the

presence of a band of high molecular weight in agarose gels without degradation and verified by PCR gene amplification[1]. For serum samples, specific biomarkers (i.e. transferrin receptor, ascorbic acid, K^+, soluble CD40L (sCD40L), adrenocorticotrophic hormone (ACTH), vascular endothelial growth factor (VEGF) and the matrix metalloproteinase-7 (MMP-7))[17] are particularly unstable and sensitive to pre-analytical variations and the ISBER Biospecimen Science Working Group have recently published the assays that can be used to assess such variation for which there is a consensus[18]. Finally, to control human PBMCs (Peripheral Blood Mononuclear Cells) cryopreserved in DMSO, cell viability is assessed after thawing[2,13], with viability values greater than 80%[19-20]. Also, the HIV/AIDS Network Coordination -HANC- proposes the assessment of PBMC performance for quality control, i.e. the number of PBMCs obtained per milliliter of collected blood[21]. In addition, granulocytes (CD15+), which contaminate PBMC isolates after prolonged blood storage, should not be present in the PBMC interphase[22].

The REDinREN (Spanish Renal Research Network) Biobank, founded in 2007, hosts a collection of samples designed for biomedical research of kidney disease and it is organized as a technical unit with quality, order and purpose criteria. Its mission is to contribute to the advance of scientific knowledge of kidney failure within the concerted action of REDinREN, affiliated with the Instituto de Salud Carlos III (RD6/0016/0002). Their activities are conducted in accordance with 14/2007 Law on Spanish Biomedical Research[23] and 1716/2011 Royal Decree[24], as well as ISBER's-International Society for Biological and Environmental Repositories-Best Practice Guide[4]. Since its inception, this biobank complies with other national laws, such as: Personal Data Protection Act[25] and the regulations for the transport of biological substances[26]. The achievements of REDinREN Biobank include its significant ongoing growth in the number and type of samples[27] received from all over Spain, the implementation of ISO 9001:2008 international standard and the accreditation of its registration in the National Spanish Registry of Biobanks. The implementation of ISO 9001:2008 standard has improved the efficiency of our biobank, by saving process execution time (by 70%), increasing the number of samples processed (by 200%) and implementing quality controls of the sample itself and of the

processes carried out[28]. Specifically, we confirmed the improvement (by 25%) in the effective management of samples transferred to research centers, one of the main processes of our biobank, resulting in higher customer satisfaction. The large increase in the number of samples processed was directly related to the improvement of the process[28]. In this work, we present the result of quality controls performed in accordance with the requirements of the above-mentioned standard, reporting the data resulting from the quality control of samples and processes. For the former, the samples analyzed were: DNA and RNA, and the viability of Peripheral Blood Mononuclear Cell (PBMC) cryopreserved in DMSO. For the latter, the process analyzed was the DNA isolation. The quality control results presented here permit us to assure that PBMC, DNA and RNA samples stored and processed at our biobank can serve as suitable sources of material for biomedical research on kidney disease.

MATERIAL AND METHODS

1 Ethical approval.

The Biobank Project was approved by the Ethical Committee of Spanish Renal Research Network's Biobank (National Register Number 0000931). Informed consent was obtained in writing from all patients after institutional approval. The studies conformed to the standards set by the latest revision of The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

2 Sample Type and collection.

The samples chosen for this work were selected from more than 76,500 stored in the REDinREN Biobank, obtained from 5,450 patients with chronic kidney disease coming from more than 70 services of Nephrology and dialysis centers from all over the Spanish territory. At present, the biobank holds samples of different types: serum, plasma, proteins, urine, blood, DNA and RNA (preserved at -80°C) and viable peripheral blood mononuclear cells (PBMC, cryopreserved at -190°C). Blood specimens were collected from renal patients into 5 different

tubes (8 milliliters each); 1 EDTA tube for plasma, 2 EDTA tubes for PBMC isolation (for DNA and protein extraction and viable cryopreserved cells), 1 tube for serum and 1 PAXgene tube (PAXgene Blood RNA Tubes, Qiagen, New Jersey, USA) for total RNA extraction[21]. The specimens were collected by personnel trained at each participating center from the antecubital area of the arm by drawing venous blood using sterile technique, transported in appropriate temperature conditions, at 4ºC, in accordance with the present law, delivered to the biobank in less than 24 hours, according to a programmed schedule with the participating center (maximum ten patients at day). Once the samples arrive, the biobank staff starts to process them without delay. The range of delay between the sample extraction and their processing by the biobank was 2 to 24 hours.

3 Implementation of quality controls.

The quality controls currently performed in the biobank are of two types:

3.1 Systematic: i.e. measurement of number of viable PBMC that were isolated from peripheral blood using Ficoll (n=2724) and purity of nucleic acids (DNA n=2328, RNA n=78)

3.2 Ad-hoc: i.e. viability of thawed PBMC (n=10), extraction of DNA from three pools of PBMC to test the DNA extraction process reproducibility (n=15) and the integrity and functionality of nucleic acids (DNA and RNA agarose gel n=48, DNA β-globin amplification n= 30, RNA RIN n=96, DNA and RNA functionality n=30).

These quality controls have been implemented on a routine basis (with established frequency). (See Table 1)

3.2.1 Sample selection criteria

The sample selection criteria for ad-hoc quality controls were the following ones: i) To analyze PBMC post-thawed viability, two selection criteria were used: samples must have been stored for at least 3 years and must have at least 5 cryovials stored from the same specimen. Two samples, each from a different year, were randomly selected for each analysis. This control was performed semi-annually, having started in 2013. ii) To prepare the 3 pools of PBMC, each one

was made on a different day with cells from 12 patients with surplus (once all relevant samples were stored, including 5 PBMC samples cryopreserved at -190ºC) of at least 8 x 10^6 cells to pool. iii) To analyze DNA integrity and functionality, the sample selection criterion used was a previous DNA extraction, with an A260/A280 ratio of 1.8-2.1. Six stored samples from different years, covering the eight-year collection period, were randomly selected for each analysis. iv) To analyze RNA integrity and functionality, the selection criterion used was a previous RNA extraction. Six samples for agarose gel and functionality and twelve samples for RIN were randomly selected for their respective analyses; samples stored in different years, covering the eight-year collection period, were used for each test. For i) to iv) the controls had been performed every four months, having started in 2013 or 2014 (see Table 1).

Table 1 summarizes, for each type of sample being tested, its implementation year, frequency, number of samples analyzed at each time, number of tests performed and total number of samples tested so far in the Biobank of REDinREN.

The number of samples of each type was approved by AENOR (Spanish Association for Standardization and Certification: "Asociación Española de Normalización y Certificación") in 2013, when the ISO 9001:2008 norm was certified, and in the first and second audit carried out in 2014 and 2015.

4 Evaluation of PBMC isolation and storage quality.

4.1 PBMCs Isolation and number of viable cells obtained.

Whole blood (from the 2 tubes anti-coagulated with EDTA) was diluted to 50% with sterile saline solution (PBS -Phosphate Buffered Saline Solution, Thermo Fisher Scientific Inc., Waltham, MA, USA). 10 ml of diluted blood was layered over 20 ml of the Ficoll gradient (Lymphocyte Isolation Solution, commercialRafer, Zaragoza, Spain). Gradients were centrifuged at 400 × g for 30 min at room temperature in a swinging-bucket rotor without the brake applied. The PBMC interface was carefully removed by pipetting. The isolated lymphocytes were washed in PBS by centrifugation at 200 × g for 10 min and the number of viable PBMC obtained was evaluated in a systematic way. For this propose, isolated

lymphocytes were counted and their viability determined by Trypan Blue exclusion, using a Countess® Automated Cell Counter (Life Technologies Ltd., Paisley, UK), as previously described[29]. Cell pellet samples were aliquoted in 6 cryovials, each containing 4 x 10^6 cells and stored at -80°C. Three samples were kept for automated DNA extraction and the other three were kept for protein extraction. Extractions are performed only when requested by a researcher. When PBMCs viability exceeded 80%, all the remaining cells were cryopreserved in liquid nitrogen at -190°C (8 x 10^6 cells per sample placed into a cryovial), in 20% Fetal Bovine Serum (FBS) supplemented medium with 10% dimethylsulfoxide (DMSO) (Sigma Aldrich, St. Louis, MO, USA).To freeze them, cryovials containing PBMCs were placed in a Mr. Frosty Freezing Container (Nalgene, Thermo Fisher Scientific Inc.) filled with isopropanol precooled at 4ºC, cooled slowly overnight to –80ºC and then transferred to liquid nitrogen.

4.2 Analysis of thawed PBMC viability.

The PBMC stored at -190ºC were thawed and cultured in 1640 RPMI medium (LONZA, Basel, Switzerland) supplemented with 10% FBS, L-glutamine (1 mM), penicillin (0.66 µg/ml) and streptomycin sulfate (60 µg/ml), at 37°C in 5% CO_2 atmosphere. No proliferative stimulus was added to the medium. Cell viability was measured by Trypan Blue exclusion at the time of thawing (time 0) and every 24 hours during the following 14 days. Each measurement for each day was performed in triplicates.

5 Analysis of total DNA extraction and DNA purity, integrity and functionality.

5.1 DNA extraction, concentration and purity.

The total DNA of the samples requested by researchers was extracted from PBMC pellets stored at -80°C. Automated extraction was performed using QIAamp DNA Mini kit (Qiagen) according to the manufacturer's standard protocol. DNA extraction was performed using a robotic workstation for automated purification of nucleic acid: QIAcube (Qiagen). DNA was quantified using a spectrophotometer (NanoPHotometer, Implen, Munich, Germany). DNA concentration was determined by measuring the absorbance at 260nm. DNA purity was

determined by the A260/A280 ratio; the A260/A280 ratio between 1.8 and 2.1 being considered acceptable[3].

5.2 DNA integrity.

DNA integrity analysis by denaturing agarose gel electrophoresis on 1% has been performed using the intact DNA untreated or treated with DNAse (27 Kunitz units, 15 min, 20-30°C) or denatured by heating (100°C, 15 min.), as specific DNA degradation negative integrity controls. The integrity of DNA retrieved was also assessed by PCR amplification of four different amplicons of the housekeeping β-globulin gene (268, 536, 989 and 1327 base pair). The PCR amplification was performed using the DNA polymerase enzyme (Biotools, Madrid, Spain), with the primers shown in Table 2. The PCR conditions were: initial denaturalization at 95° C for 3 min., followed by 40 denaturation cycles at 95°C for 1 min., annealing at 55° C for 1 min., 72° C extension for 1.5 min., and a final extension at 72°C for 5 min. The PCR products obtained were analyzed by gel electrophoresis on a 1% agarose gel. Gels were stained with RedSafe™, Nucleic Acid Staning Solution (20000x) (Promega, Madison, WI, USA). DNA of acceptable quality had at least three bands of ß-globulin amplified from DNA[1].

5.3 DNA functionality by quantitative PCR.

Endogenous β-actin and GAPDH genes were amplified by qPCR with LC FastStart DNA Master SYBR Green I Kit (Roche, Mannhein, Germany), in direct DNA (250ng in each reaction, carried out in triplicate) samples. The pairs of primers used were: human GAPDH: 5´-TCC ACT GGC GTC TTC ACC- 3´ (forward) and human GAPDH: 5´-GGC AGA GAT GAC CCT TTT- 3´ (reverse); human β-actin: 5´-TCA CCC ACA CTG TGC CCA TCT ACG A- 3´ (forward) and human β-actin: 5´-CAG CGG AAC CGC TCA TTG CCA ATG G- 3´(reverse). qPCR was performed employing the 7500 Fast real-time PCR System and 7500 Fast sequence detection software v1.3.1 (Applied Biosystems, CA, USA) as per the following cycling conditions: 50ºC for 2min and 95ºC for 10 min, followed by 45 cycles of 95ºC for 15 sec, and 60ºC for 1min.

6 Analysis of total RNA extraction and RNA purity, integrity and functionality.

6.1 RNA extraction, concentration and purity.

Total RNA extraction was conducted using peripheral blood samples stored in PAXgene tubes (PAXgene Blood RNA Tubes, Qiagen), collected from each participating center. At the biobank, automated extraction was performed using the PAXgene® Blood RNA Kit (Qiagen), according to the manufacturer's instructions. RNA extraction was performed using a robotic workstation for automated purification of nucleic acid: QIAcube. RNA samples were preserved at -80°C. When samples were requested, RNA concentrations were systematically measured with a spectrophotometer (NanoPHotometer, Implen). RNA purity was also systematically determined by measuring the A260/A280 ratio.

6.2 RNA integrity.

The RNA integrity was analyzed by determining the RNA Integrity Number (RIN), using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), in the Department of Genomics of the PCM Foundation, Universidad Autonoma de Madrid, Spain. The RIN, an algorithm based on a selection of features resulting from data obtained from an electropherogram of a given sample[30]. RIN is a scale of RNA quality with RIN 1 denoting completely degraded RNA, RIN 10 denoting completely intact RNA and a RIN 7 value is regarded as a threshold above which samples are suitable for most applications[1,30-31].

6.3 RNA functionality by quantitative RT-PCR.

cDNA was obtained from RNA (2µg) by using the High Capacity cDNA Reverse Transcription Kit. Then, endogenous β-actin and GAPDH genes were amplified by qPCR in cDNA (250ng in each reaction, carried out in triplicate), as described for DNA, using the same probes. As a sample selection criterion, RNA had been previously extracted and the sample number and collection period were the same as for DNA.

7 Quality control of the DNA extraction process.

After isolation, the selected PBMC were pooled, stored in cryovials (4 x 10^6 cells) and frozen at -80ºC until their use. DNA concentration and purity analysis on the 3 different PBMC pools was conducted each 4 months as described and the coefficients of variation (CV), calculated as CV= (standard deviation (SD)/mean)*100, obtained from the different measures performed to the same pool were calculated.

8 Statistical Methods

Descriptive statistics were used to show the data concerning the different distribution values. The normality of the distributions was tested by the Kolmogorov-Smirnov test. For normal distributions, to compare the delay in processing time values for the PBMC yields and the DNA ratios, the Student's paired *t*-test was performed. For normal distributions, to compare the differences among the time of storage of the samples, ANOVA was used with no additional tests. The normal distributions were represented as mean ± SD. For non-normal distributions, assigning statistical significance to the differences between the storage times and the delay to processing times, the Kruskal-Wallis test or Mann-Whitney U test were used. The non-normal distributions were represented as median plus an interquartile range (IQR). Statistical significance was set at $p<0.05$.

RESULTS

Quality control of viable PBMC isolation

PBMC isolation was evaluated by examining the number of viable cells obtained per milliliter of whole blood. The mean of viable PBMC obtained remained stable without significant differences from year 2010 to 2015 (p = 0.3637) (Figure 1). The mean total number of viable cells isolated was 3.17 ± 1.12 x 10^6 PBMC/ml of blood from 2,724 specimens analyzed, which were the ones admitted to the biobank in those years (year 2010 (n = 181), 2011 (n = 868), 2012 (n = 902), 2013 (n = 359), 2014 (n = 255) and 2015 (n = 159). Moreover, although viable PBMC mean number obtained in samples with less than 4 h pre-centrifugation delay was higher

when compared to the 24 h delay group, the difference was not statistically significant (mean 4 h: 3.36 ± 1.12, n=523; mean 24 h: 3.01 ± 1.18, n=2,201. p = 0.469, *t*-test).

Quality control of recovery and viability of Peripheral Blood Mononuclear Cells stored at -190ºC.

Figure 2 represents the viability of the ten thawed samples. As shown on day 0, the viability was 96.5% (IQR 94.75-97.25), which indicates that the freezing process did not affect cell viability. Values above the horizontal line, which indicates a cell viability of 80% -minimum acceptable viability threshold-, were recorded on days 1 to 14. At the 14th day, viability was observed to descend to 80%. The median percentage of post-cryopreservation viable cells recovery of these ten samples of PBMC was 84.5% (IQR 78.4–100.0). When we evaluated the percentage of PBMCs that were still viable after 14 days in culture, and compared it between aliquots of PBMCs that had been in storage for the longest period of time (years 2007-2009; 85%, IQR 83-87.5) and the shortest period of time (years 2010-2012; 83%, IQR 80.5-83) the difference fell just short of being statistically significant (p = 0.056, Mann-Whitney U test). This analysis indicates that long-term storage time may not affect the cryopreserved PBMC viability. In addition, the percentage of live cells after 14 days post-thawing was not significantly different between the samples which were collected and processed with a delay of less than 4 h and those of less than 24 h (p = 0.383, Mann-Whitney U test).

Quality control of DNA samples stored at -80ºC.

To evaluate the quality of DNA samples, we analyzed three parameters: purity, integrity and functionality, measured as gene amplification ability. Figure 3A shows the purity of the DNA isolated from 2,328 samples analyzed, which are the total number extracted to date, grouped by year of storage. The mean A260/A280 ratio value was 2.05 ± 0.14and the storage time is not affecting the DNA purity (p = 0.063). In addition, the mean DNA ratio was not significantly different between the samples which were collected and processed with a delay of less than 4 h and those of less than 24 h (mean 4 h: 2.025 ± 0.21, n=498; mean 24 h: 2.059 ± 0.18, n=1,830.

p=0.338, *t*-test). Figure 3B shows the DNA integrity in two representative samples of the 48 analyzed to date, indicated by the presence of a single high-molecular weight band in the intact DNA (untreated), in comparison with negative controls after DNAse treatment and after being heated, with no band or a very diffuse band being observed in either of the latter two cases, as expected. We have found some partial degradation in the 4.2% of extracted DNA samples (2 of 48 samples), following electrophoresis (Figure 3B, Sample 2). These data were completed with the PCR amplification of four β-globulin gene fragments. Figure 3C shows four amplified fragments of a representative sample of the 30 analyzed to date, with sizes ranging from 268 to 1327 bp, which correspond to high quality DNA (without being fragmented). DNA of acceptable quality with at least three bands of β-globulin amplified was obtained in 26 of the 30 samples analyzed to date (76.7% (n=23) four bands; 10% (n=3) three bands; 13.3% (n=4), two bands). Finally, DNA retrieved functionality was assessed by β-actin and GAPDH constitutive genes amplification by qPCR (Figure 3D and E). The median Ct values (number of cycles required to reach the detection threshold) of the 30 samples studied to date were 19.75 (IQR 19.25-21.32) for β-actin and 22.37 (IQR 21.91-23.18) for GAPDH, which demonstrates that it can be used for genome analysis. As observed, storage time is not affecting DNA functionality (*p*=0.239 for β-actin and *p*=0.969 for GAPDH, Kruskal-Wallis test). In addition, the median Ct value was not significantly different between the samples which were collected and processed with a delay of less than 4 h and those of less than 24 h (*p*=0.440 for β-actin and *p*=0.134 for GAPDH, Mann-Whitney U test).

Quality control of RNA samples stored at -80ºC.

The quality control of stored RNA samples was likewise performed by assessing the following parameters: purity, integrity and amplification ability. Figure 4A shows the RNA purity of the 78 samples extracted to date, grouped by year of storage. The median A260/A280 ratio value was 2.04 (IQR 1.96-2.07) and, as observed, storage time is not affecting RNA purity (*p*=0.089, Kruskal-Wallis test). Moreover, the median RNA ratio was not significantly different between the samples which were collected and processed with a delay of less than 4 h and those of less

than 24 h (p=0.374, Mann-Whitney U test). The RNA integrity was determined by the assessment of RIN. As shown in Figure 4B (Left Panel), the mean RIN value for 2007-2015 period was between 7.3 and 9.1 (8.19 ± 0.92); which demonstrates that long-term storage time is not affecting the integrity of stored RNA in the 96 samples analyzed to date (p=0.092). Very good RIN numbers ≥ 7 were obtained in 87 of 96 samples. Finally, Figure 4C and D show β-actin and GAPDH constitutive genes amplified by RT-qPCR. The median RIN value was not significantly different between the samples processed with a delay of less than 4 h and those of less than 24 h (p=0.079, Mann-Whitney U test). The median Ct values of the 30 samples studied to date were 20.04 (IQR 18.2-20.86) for β-actin and 21.0 (IQR 19.0-22.71) for GAPDH. These results confirm that these samples can be used for gene expression analysis. Moreover, it can be observed that storage time is not affecting RNA functionality (p=0.639 for β-actin and p=0.773 for GAPDH, Kruskal-Wallis test). Finally, the median Ct value was not significantly different between the samples which were collected and processed with a delay of less than 4 h and those of less than 24 h (p=0.477 for β-actin and P=0.373 for GAPDH, Mann-Whitney U test).

Quality control of DNA isolation processes.

Finally, we performed the quality control of one of the most widely used processes, which is routinely carried out in the biobank. We assessed DNA isolation process by using 3 PBMC pools. Table 3 shows the mean concentration and purity values of the different measures performed to the same pool, and the CV of the measurements obtained, which demonstrates the reproducibility of the process. The lows CV for the ratio, especially as an indicator of the purity of DNA retrieved are particularly notable.

DISCUSSION

The aim of the present study was to determine and to present the results of the quality controls performed on the PBMC, DNA and RNA samples stored in the REDinREN Biobank, implemented according to ISO 9001:2008 standard, including quality controls of samples and processes. The results indicate that these samples obtained and preserved in this biobank have

the suitable quality and reproducibility to be used in biomedical research, using both classic analysis methodology and new high performance technology.

First, the high number of viable PBMC obtained largely depends on the effective isolation from whole blood samples using the Ficoll method[29]. The results of PBMC isolation carried out at this biobank indicate an excellent quality, unaffected by the year when the process was performed or the number of samples processed.

Moreover, the quality control of cryopreserved cells yields good levels of PBMC post-thaw viability; which guarantees that the freezing, cryopreservation and thawing process is being developed in a correct way. Efficient cryopreservation of cells offers many advantages to the research community[32]. This technique makes it possible that samples from the same donor banked over time can be processed simultaneously, allowing greater inter- and intra-laboratory control and reducing costs. It was previously shown that the viability of cryopreserved PBMCs has tremendous effects on the results of functional assays[32], so that at least 80% viability is necessary to generate conclusive responses to antigens and mitogens. These PBMC can also be efficiently used for immunomagnetic separation of cells, cytokine production and flow cytometer analysis[33-34].

The limit of the length of time a PBMC sample can be stored in a biobank has not yet been determined, although numerous quality control studies approach this analysis on different types of samples stored[13,19]. In this study, samples are chosen at random from different years of storage. When we compared the percentage of PBMCs that were still viable after 14 days in culture, the analysis indicates that long-term storage may not affect the PBMC viability. We observed slightly higher viability values of the PBMCs that had been storage for a longer period of time. The almost statistically significant difference may be caused by the increased number of samples processed (deposited or transferred) during the more recent years studied. Due to the low number of samples analyzed, this result could be confirmed with a larger set of samples. We have now improved the process, protocols and staff training, with the changes that we performed during achievement of the ISO 9001:2008 standard implementation. These observations are of special importance for the long-term future of the REDinREN Biobank since

one of its objectives is to utilize biospecimens associated with clinical data to design and perform prospective studies.

Moreover, it has been shown that time from venipuncture to PBMC isolation and cryopreservation may affect the cell quantity, recovery, viability, white blood cells subpopulation distribution, gene expression, and lymphoblastoid cell lines transformation[35]and also have impact in the viability and performance of T cells in downstream immunological assays[36-37] and in the cytotoxic activity of natural killer cells[38]. In addition, it has also been shown that a 24-h delay between blood collection and processing, especially at 4ºC, significantly increases granulocyte contamination, which may inhibit T cell function[22]. Due to these findings, some authors recommend the processing of blood samples for PBMC isolation within shorter time periods (e.g. 8 hours)[22,35-40]. At that time, our biobank was not able to assess potential granulocyte contamination of the PBMC isolates. However, in the present study, we analyzed samples from a wide range of processing delay (i.e. 2-24 hours) and we statistically compared the results between samples processed within less than 4 h or less than 24 h of collection. Although viable PBMC number obtained in samples with less than 4 h pre-centrifugation delay was higher when compared to the 24 h delay group, the difference was not statistically significant. Likewise, statistical significance could not be assigned to any other comparisons we made between the two delay to processing cohorts. Due to the relatively low number of samples analyzed, this result may be confirmed on a future larger set of samples and further studies may address the optimization of biospecimen processing methods to obtain high cell yields, the adequate subpopulation composition and improved cell viability for downstream applications in our stored samples.

With regard to the analysis of samples of nucleic acids (DNA and RNA), the values obtained for the A260/A280 ratios indicate that both materials have an acceptable degree of purity, suitable for most applications[8-9,41]. The absorbance A260/A280 ratios of most samples were ranged between 1.8 and 2.1, which indicates that both nucleic acids obtained reach high purity levels. In addition, DNA and RNA A260/A280 ratios values did not show a significant difference related to stored year, which confirms long storage time does not affect their quality.

Indeed, the vast majority of the samples analyzed presented good ratios, within normal values[42-43], with a small percentage of samples (9.4% for DNA and 6.1% for RNA) deviating from the acceptable range. In the case of DNA analysis, the normal range of the A260/A280 ratio is between 1.8 and 2.1. A ratio higher than 1.8 is generally accepted as an indicator of pure nucleic acids whereas ratios lower than 1.8 may indicate contamination by proteins or other contaminants that absorb at or near 280 nm, or may also be due to the effect of several solvents used during the extraction of nucleic acids[3,42]. On the other hand, co-eluting RNA can become a "contaminant" of unknown magnitude in a DNA extraction, leading to an overestimation of DNA yield[44]. An alternative solution could be to use spectrofluorometry (in addition to spectrophotometry) to quantify the nucleotides routinely. In our protocol, we decided to do not include RNase digestion, because we do not want to cross-contaminate either our laboratory or the QIAcube. For this reason, the data presented here are an estimate of the DNA concentration. In addition, contamination of the sample of DNA with RNA does not impair subsequent use in the majority of downstream applications. However, the use of DNase digestion is mandatory in the case of RNA extraction to prevent erroneous amplifications. In RNA analysis, a A260/A280 ratio higher than 1.8 is usually accepted as an indicator of a pure RNA, relatively free of proteins[43]. We have not been able to identify the cause of the unacceptable purity values observed in the samples mentioned above; however, they are within the expectable margin of errors when handling or transporting samples. In addition, we have implemented corrective actions for these non-conforming results (Table 4), which allowed a 10% reduction in the DNA or RNA samples deviated from the acceptable range.

The absorbance readings give no indication related to the physical integrity (lack of fragmentation) of the sample. A traditional method that can be used to assess nucleic acids integrity is agarose gel electrophoresis[42, 45]. In our results, the intact genomic DNA appears as a compact high molecular weight band with no or little low-molecular-weight material.

Additionally, taking into account that the RNA is the most easily degradable component of biological samples, among other reasons, due to endogenous ribonucleases[46], we assessed its integrity by calculating the RNA Integrity Number (RIN)[31]. In our study, only 9 of the 96

samples analyzed presented RIN values of less than 7; we have implemented corrective actions (Table 4).

Our results do not show a decrease in RIN values with increasing storage time of the specimens in the biobank. Several studies have examined the RIN variation associated with storage time. In 2012, Bao et al. studied RNA from tissues collected over a 40-month period and obtained RIN values higher than 7 for all the samples analyzes[47]. Recently, Hebels et al. assessed RIN values in blood samples stored during a 4-19 year period at -80º C in Sweden, and during an 11-19 year period in liquid nitrogen in Italy, and found no observable systematic adverse effects of storage time[48]. In 2013, Andreasson et al. analyzed RNA from 153 endocrine tissue samples stored for over almost three decades; the results suggest that RNA quality from tissues is not adversely affected by storage in well-monitored and maintained -80ºC freezers, which are capable of excellent preservation of high-quality RNA for decades. These findings challenge the arguments that claim that using liquid nitrogen for storage is always better and support the idea that -80ºC freezers are equally suitable for long-term preservation of tissue samples, with additional benefits resulting from environmental hazards[11]. In REDinREN Biobank, where RNA is preserved in freezers at -80ºC, the RIN number of samples stored is routinely assessed in order to ensure that the standards of quality required for stored material are uniform throughout the time.

The utility of DNA or RNA retrieved from tissues for subsequent molecular applications can also be assessed by PCR amplification of a specific sequence. Nucleic acids with little or no PCR or RT-qPCR product indicate tissue degradation and poor quality[6]. Our results showed acceptable Ct values, which is a good indicator of the suitability of the material for subsequent use in biomedical research[35].

The quality control of the processes was also studied. The automated DNA extraction demonstrated the reproducibility and reliability of the standardized protocols and manipulations performed. The coefficient of variation obtained for A260/A280 ratio was very low, which is significant as this is indicative of the consistent purity of the DNA retrieved. Low coefficients of variation of DNA concentration also ensure intra-assay reproducibility of this method.

Up to the present, we have not performed either systematic or routine quality controls of plasma and serum samples stored in our biobank. So far, the scientific community has not reached a consensus to identify the best biomarkers to determine plasma and serum quality. The main problem is that it has not been possible to perform a specific test in a prospective way because the quality of plasma and serum is strongly linked to the research program. In this sense, biobanks, including our REDinREN Biobank, are a valuable tool, because they have competence to develop their own research, which enables them to optimize processes and find easy detection biomarkers to ensure the quality of all sample types. In addition, nine peer-reviewed publications have resulted from samples in our biobank, which demonstrates that the samples are of quality that is amenable to downstream research[49-57].

Finally, it should be noted that, taking into account the special features of REDinREN Biobank, which contains samples from throughout Spain that must be transported to our center before being processed, our results support the idea that the construction of large sample cohorts from multiple centers can be carried out as long as strict controls are implemented over sample collection, transportation, reception and processing. Table 5 summarizes the difficulties related to multisite sample collection and the solutions implemented in REDinREN so as to better coordinate the process and the training of health technical personnel and to closely supervise all the partners involved in sample collection and delivery, including the establishment and implementation of common Technical Instructions. These results have a special relevance, taking into account that there are currently very few studies reporting on quality controls on specimens coming from different centers.

Supplementary Figure 1 summarizes the role of the REDinREN Biobank Quality Management System to monitor, assess and identify the customer's requirements, to analyze conforming or no-conforming results and to implement corrective or improvement actions.

CONCLUSION

Typically, the collection of significant numbers of human biological samples and associated quality information involves a major effort in planning, construction, and finally operation, which consumes a significant amount of time and which slows down the development of experimental research. Hence, the promotion and implementation of biobanks, which facilitate the researcher's access to quality samples (and associated data) with the proper ethical and legal safeguards for the donor, represent a major advance in shortening the time that normally elapses between research and the application of its results and in improving the effectiveness of research. The appropriate control that ensures the quality of the samples offered and their adequate long-term preservation in repositories is crucial. Maintenance and improvement of quality control are part of the REDinREN Biobank's policy of ongoing improvement.

The results presented in this study show that PBMC, DNA and RNA samples stored at REDinREN Biobank meet the necessary quality standards to be used in current biomedical research and that the methodology used complies with the requirements to ensure the long-term preservation of the specimens.

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	Mean concentration, ng/µl (SD)	Concentration CV (%)	Mean A260/A280 ratio (SD)	A260/A280 ratio CV (%)
Pool 1	86.75 (10.35)	11.93	2 (0.025)	1.25
Pool 2	102.50 (9.82)	9.58	2.03 (0.02)	1.18
Pool 3	115.11 (8.30)	7.21	1.95 (0.01)	0.41

Table 3: Quality control of the DNA extraction process. The table shows the mean ± SD values and coefficients of variation (CV) = (SD/mean)*100 of DNA concentration and the A260/A280 ratios, which were measured 5 times to date, from 3 different pools of PBMC, in accordance with our usual Protocol.

PROCESS	PROBLEM	CORRECTIVE ACTION
Ficoll recovery of viable PBMC.	Sample hemolysis	Hemolyzed samples are excluded from quality control analysis. Formulation of acceptance criteria.
	Less than 80% viability	No PBMC cryopreservation. Formulation of acceptance criteria.
Nucleic acid extraction	A260/A280 purity ratio deviates from acceptable criteria range.	Vessels containing buffers are changed whenever an extraction is performed
RNA Integrity Number (RIN) determination	Samples with low RIN values, less than 7.	They are marked. The researcher is notified when these samples are delivered.

Table 4: Summary of problems and corrective actions to improve the process of sample management implemented in REDinREN Biobank. (PBMC) Peripheral Blood Mononuclear Cells.

DIFFICULTIES	SOLUTION
Different sample collection tubes (type and volume)	Implementation of common Technical Instructions.
Different health technical personnel to extract blood	-Implementation of common Technical Instructions. -Training courses addressed to biobank technicians.
Samples that are not suitably refrigerated	Sending appropriate refrigeration equipment and instructions for use.
Delay in sample delivery or receipt	Coordinating the service of removal, shipment and receipt of the sample.
Broken tubes	Providing all centers with a sample cylinder for protection during shipping.
Problems caused by couriers' delays or failure to collect the specimens	Changing the courier company and setting a new schedule.

Table 5: Summary of difficulties and solutions implemented in REDinREN Biobank related to multisite collection of samples. All samples were shipped to a central site using common Technical Instructions.

FIGURE LEGENDS

Figure 1: Quality of PBMC (Peripheral Blood Mononuclear cells) isolation from peripheral blood. Analysis of the performance of the PBMC isolation technique expressed as the number of viable cells obtained per milliliter of whole blood, associated with their year of isolation. Total number of specimens processed: 2,724. Plots represent the mean ± SD values for each year.

Figure 2: Viability of Peripheral Blood Mononuclear cells (PBMC) cryopreserved in 10% DMSO. Viability of cells thawed and cultured for 14 days, measured by Trypan Blue exclusion test from Day 0 (thawing day) to Day 14. The % of live cells from ten independently thawed samples was achieved. Dots represent individual values from all the samples studied. Bars represent the median and interquartile range values for each day. The horizontal line represents 80% of cell viability.

Figure 3: Quality of DNA isolated from PBMC by automated QIAcube (Qiagen) system. Panel A: isolated DNA ratio of absorbance at 260nm/280nm (A260/A280) of the 2,328 samples analyzed associated with their year of storage. Plots represent the mean ± SD values for each year. Panel B: representative image of gel agarose electroforesis of 2 samples of genomic DNA in the following three assumptions: CT (untreated DNA control), 1 (DNA treated with DNAse) and 2 (DNA after being heated at 100º C for 15 minutes). Panel C: representative image of a very good DNA sample assessed by PCR amplification of β-globulin gene fragments that presents four PCR amplified products in four independent PCR reactions. Panel D and E: Ct values of 30 DNA samples associated with their year of storage, obtained by qPCR amplification of endogenous β-actin (D) and GAPDH (E) genes. Dots represent individual values from all the samples studied. Bars represent the median plus interquartile range values for each year.

Figure 4: Quality of RNA isolated from peripheral blood by PAXgene system. Panel A: isolated RNA ratio of absorbance at 260nm/280nm (A260/A280) of the 78 samples analyzed associated with their year of storage. Panel B: RIN (RNA Integrity Number) values obtained for

96 samples associated with their year of storage. On the right panel, an electropherogram representative of a sample of total RNA with a good RIN value of 9.9 (upper panel) and a poor RIN value of 5.7 (bottom panel) with no distinct bands visible, can be observed. The horizontal line represents a RIN value of 7. Panel C and D: Ct values of 30 RNA samples associated with their year of storage, obtained by RT-qPCR amplification of endogenous β-actin (C) and GAPDH (D) genes. Panel A to D: dots represent individual values from all the samples studied. Bars represent the median plus interquartile range values for each year.

FIG 1

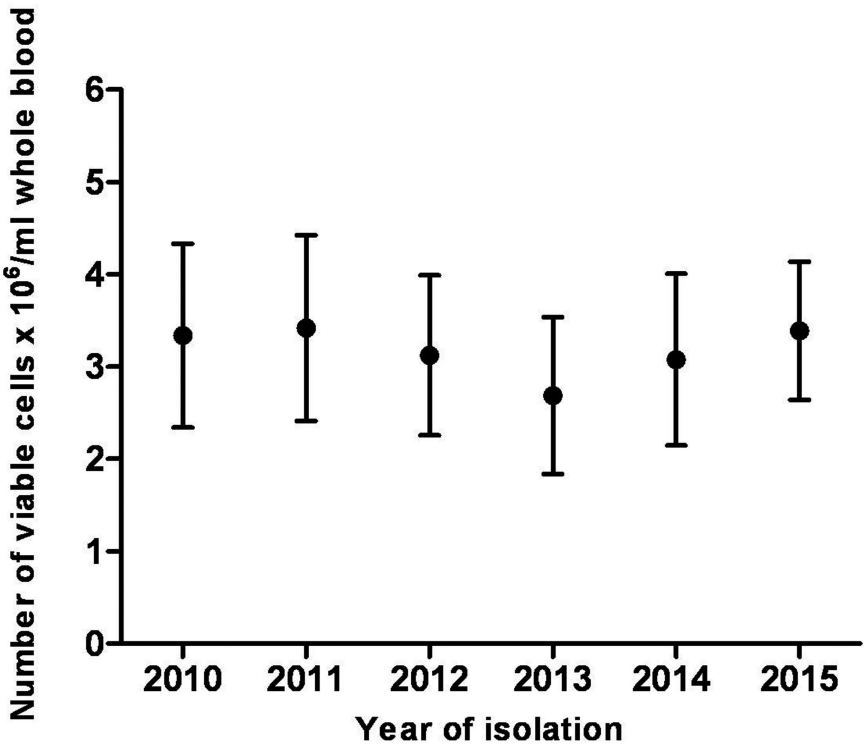

Number of viable cells x 10^6/ml whole blood
6
5
4
3
2
1
0
2010
2011
2012
2013
2014
2015
Year of isolation

FIG 2

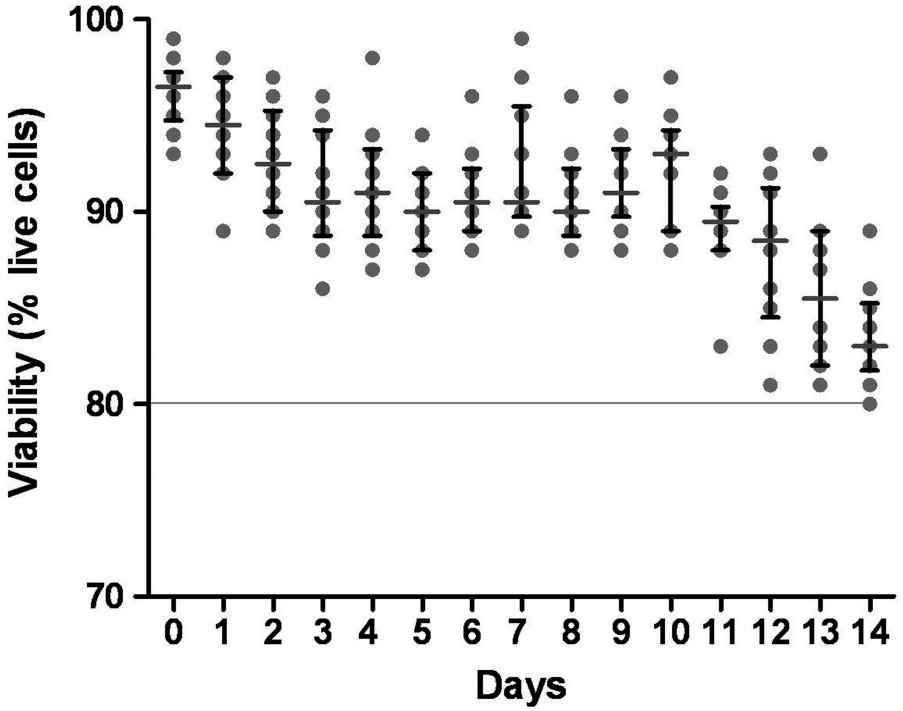
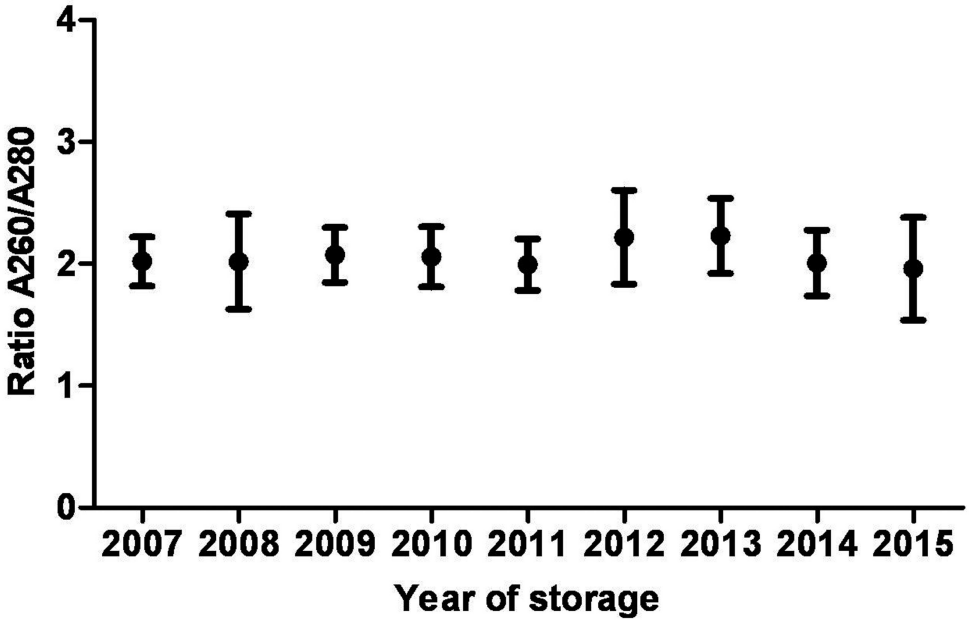
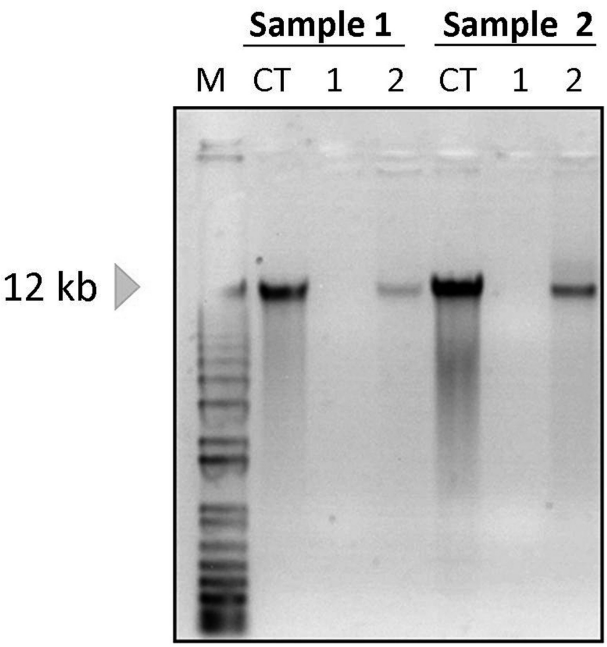

Viability (% live cells)
100
90
80
70
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Days

FIG 3

A


Ratio A260/A280
0
1
2
3
4
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

B


Sample 1
Sample 2
M CT 1 2 CT 1 2
12 kb

C

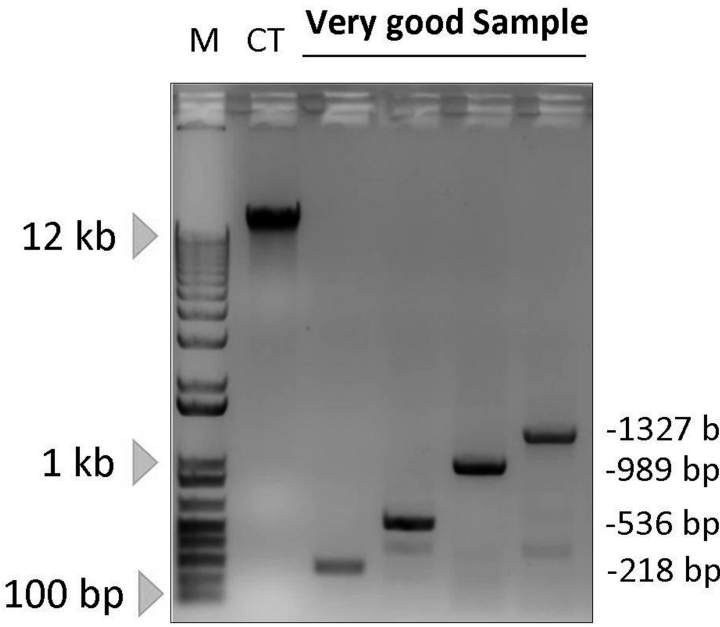
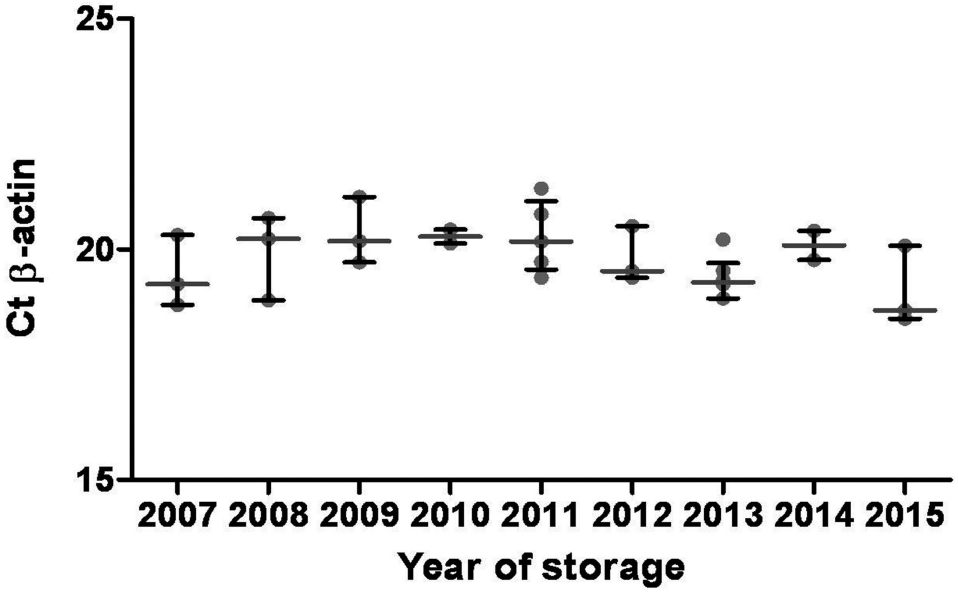

M CT
Very good Sample
12 kb
1 kb
100 bp
-1327 b
-989 bp
-536 bp
-218 bp

FIG 3

D


25
20
15
Ct β-actin
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

E

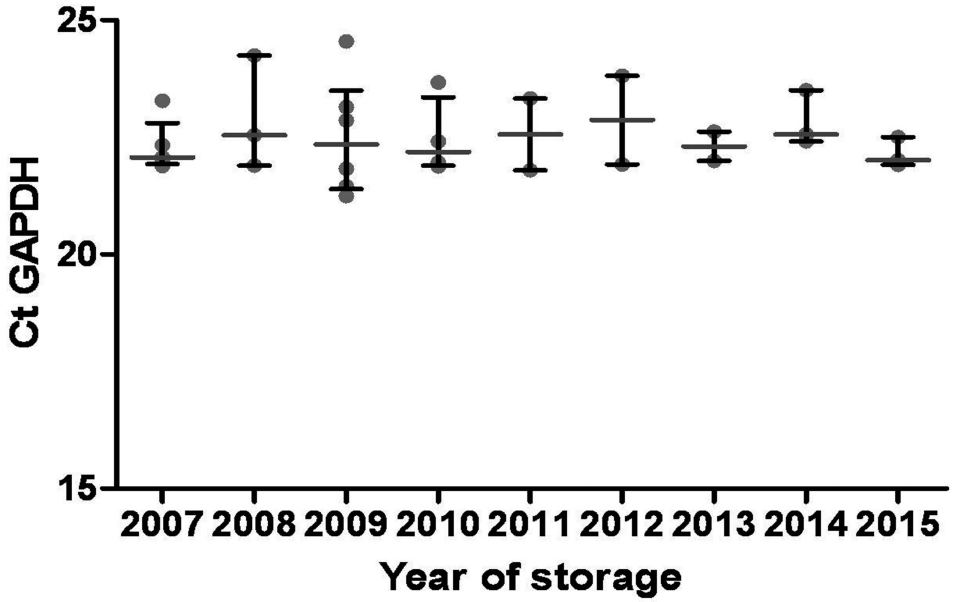
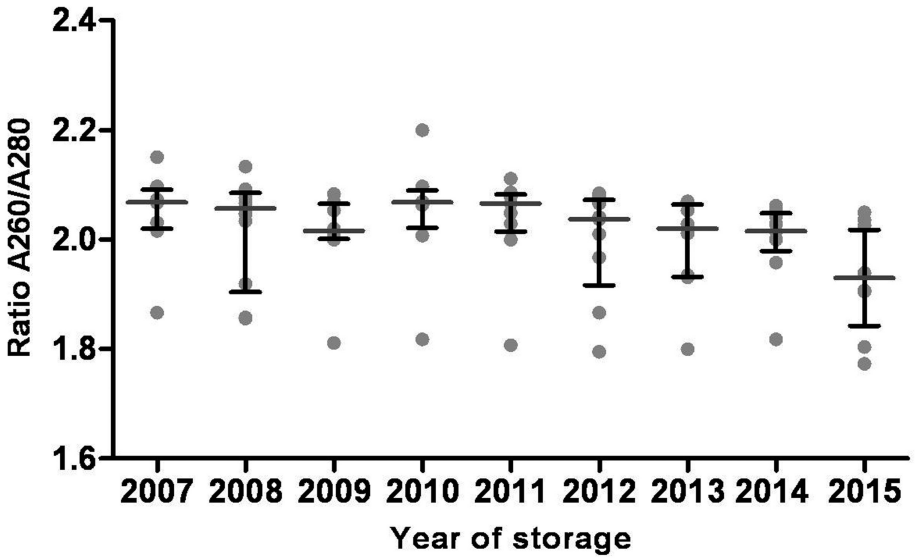
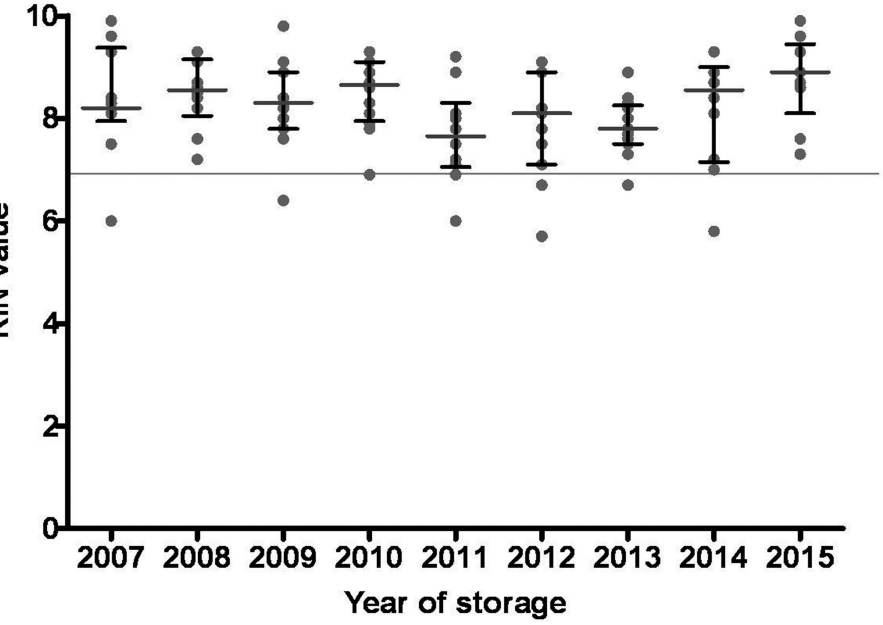

25
20
15
Ct GAPDH
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

FIG 4

A


Ratio A260/A280
2.4
2.2
2.0
1.8
1.6
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

B


RIN value
10
8
6
4
2
0
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

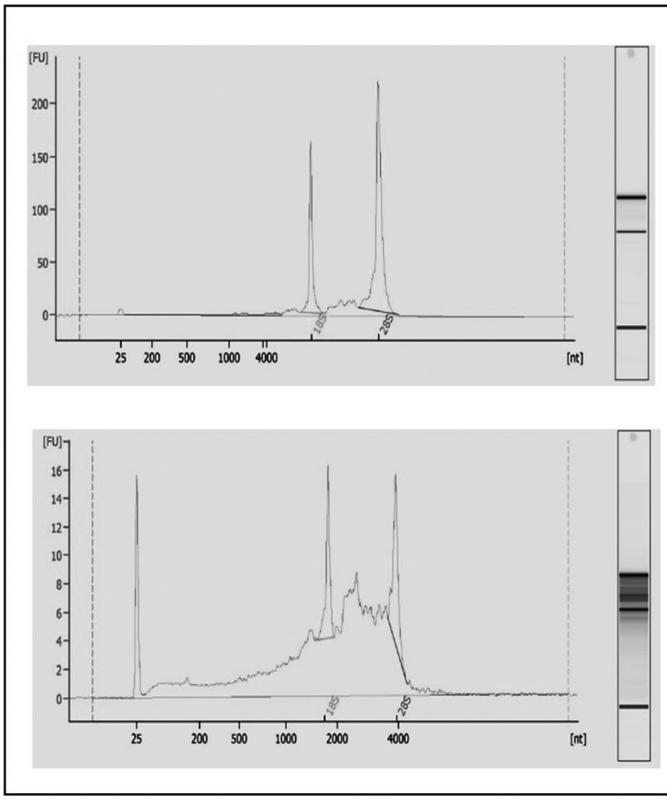
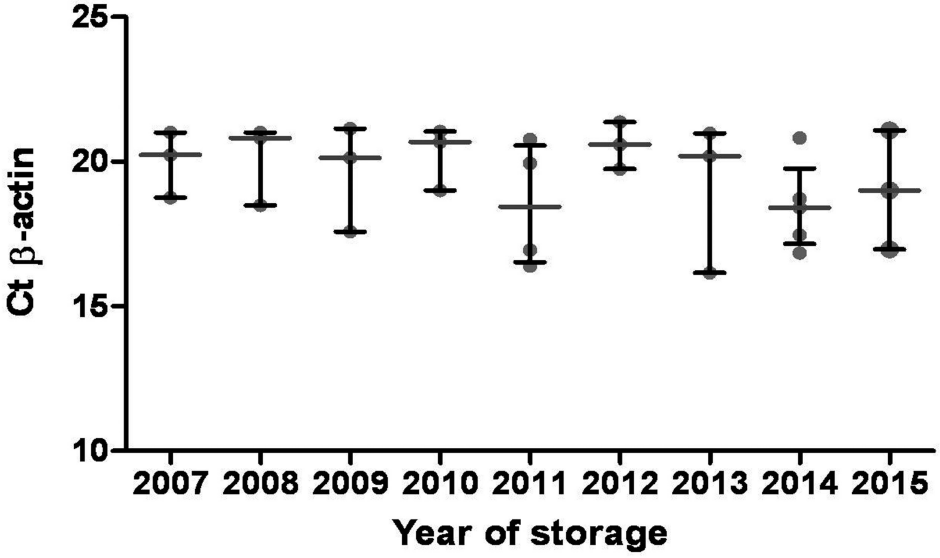
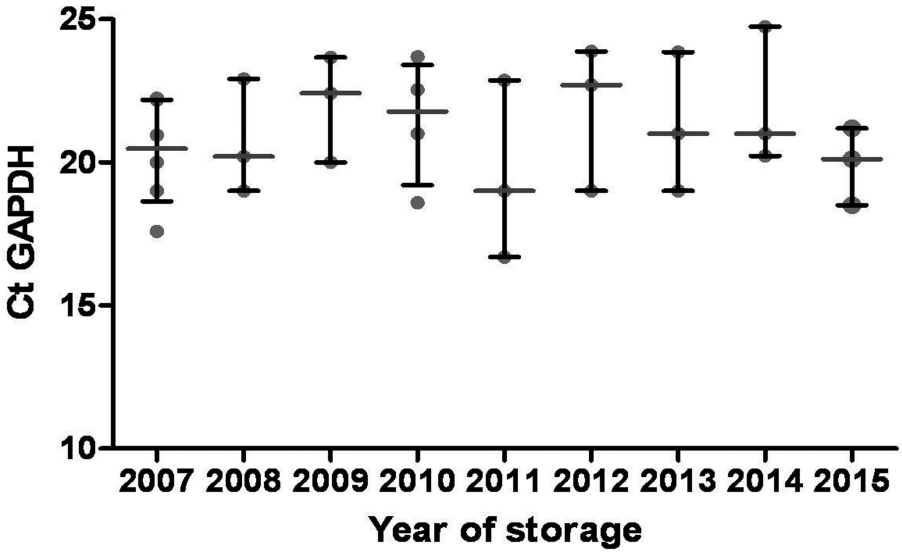

[FU]
200
150
100
50
0
25 200 500 1000 4000
[nt]
18S
28S
[FU]
16
14
12
10
8
6
4
2
0
25 200 500 1000 2000 4000
[nt]
18S
28S

FIG 4

C


25
20
15
10
Ct β-actin
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage

D


25
20
15
10
Ct GAPDH
2007 2008 2009 2010 2011 2012 2013 2014 2015
Year of storage